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The effects of partial thiamin deficiency and oxidative stress (i.e., glyoxal and methylglyoxal) on the levels of α-oxoaldehyde plasma protein adducts in Fischer 344 rats

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Abstract **We hypothesized that in marginal thiamin deficiency intracellular α-oxoaldehydes form macromolecular adducts that could possibly be genotoxic in colon cells; and that in the presence of oxidative stress these effects are augmented because of decreased detoxification of these aldehydes. We have demonstrated that reduced dietary thiamin in F344 rats decreased transketolase activity and increased α-oxoaldehyde adduct levels. The methylglyoxal protein adduct level was not affected by oral glyoxal or methylglyoxal in the animals receiving thiamin at the control levels but was markedly increased in the animals on a thiamin-reduced diet. These observations are consistent with our suggestion that the induction of aberrant crypt foci with marginally thiamin-deficient diets may be a consequence of the formation of methylglyoxal adducts.**

Keywords: α-Oxoaldehydes; Methylglyoxal; Glyoxal; Oxidative stress; Thiamin; Protein adducts

1. Introduction

Thiamin (vitamin B_1) in its diphosphate form (TDP) is an important coenzyme for transketolase (TK), pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and the branched-chain α-ketoglutarate dehydrogenase complex. These enzymes are involved in the maintenance of NADPH levels and carbohydrate metabolism in the cell (Scheme 1) [1,2]. Thornalley et al. [3] have shown that accumulation of methylglyoxal (MG, an α-oxoaldehyde) and triose phosphates in human red blood cells incubated under hyperglycemic conditions can be prevented by thiamin. Moreover, Thornalley's group has recently shown that high dose thiamin and benfotiamine (lipophilic derivative of thiamin) therapy in diets administered to streptozotocin induced diabetic rats prevented incipient diabetic nephropathy induced by reactive α-oxoaldehyde [4].

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Abbreviations: MG, methylglyoxal; TK, transketolase; TPP, thiamin pyrophosphate; RBC, red blood cells; ACF, aberrant crypt foci; AGEs, advanced glycation end-products

MG is formed by the non-ezymatic elimination of phosphate from the triosephosphates glyceraldehyde-3-phosphate and glycerone phosphate, as well as enzymatically from dihydroxyacetone phosphate catalyzed by the allosterically regulated MG synthase [5,6]. It is also formed via ketone body metabolism from acetone, and by the metabolism of threonine [7]. Glyoxal (MG derivative), however, is a product of lipid peroxidation, ascorbate autoxidation, oxidative degradation of glucose and glycated proteins [8]. MG and glyoxal are bifunctional alkylating agents that react with free amino and thiol groups of biomolecules, resulting in the formation of advanced glycation end-products (AGEs). Glyoxal and MG react non-enzymatically and reversibly with lysine, arginine and cysteine residues of proteins [9,8]. Irreversible reactions with lysine and arginine result in glyosylamine protein crosslinks and imidazolone (Scheme 2) derivatives, respectively [10].

Although glyoxal and MG have not been classified as carcinogens [11], they appear to be tumor promoters and have been shown to be direct mutagens in several cellular models [12]. In studies done with rat liver microsomal fractions (S9) or thiol compounds, the mutagenic activity of these compounds was abolished [13,14]. Therefore, the mutagenic expression of these compounds may be ameliorated by cellular antioxidant and metabolic capacity.

MG and glyoxal are detoxified endogenously primarily by the glyoxalase system, which converts glyoxal to glycolate in the presence of glutathione (GSH) [15]. Another minor detoxification pathway for glyoxal is catalyzed by oxido-reductases. The family of oxido-reductases involves the enzymes usually represented as ALR1, ALR2 and ALR3, and are called aldehyde reductase, aldose reductase and carbonyl reductase, respectively. These enzymes have a broad substrate specificity, are located in the cytosol and require NADPH or NADH to a lesser degree as a co-factor [16]. Under conditions of oxidative stress, the level of GSH decreases and this impairs glyoxal and MG detoxification by the glyoxalase system [15]. On the other hand thiamin deficiency affects the metabolism of these species via the oxido-reductase pathway due to a decrease in NADPH levels that eventually causes a decrease in GSH levels [17]. Therefore, it appears that oxidative stress and thiamin deficiency have an overlapping requirement for NADPH and GSH.

Recently, we reported that thiamin deficiency in Fischer 344 rats induced the formation of aberrant crypt foci (ACF, putative precursors of colon cancer) [18] in the absence of clinical beriberi symptoms. Furthermore, thiamin deficiency in mammalian cells in vitro markedly increased hepatocyte susceptibil-

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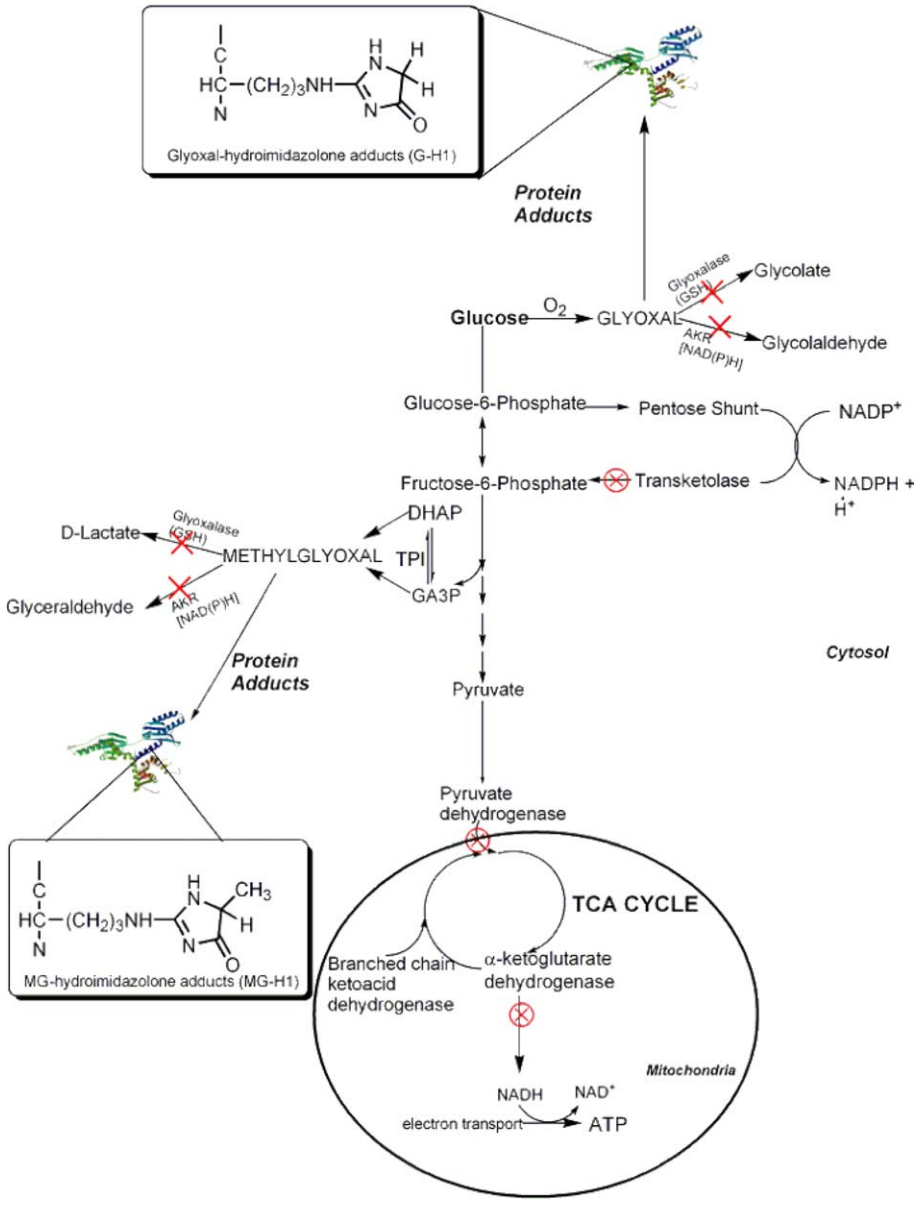


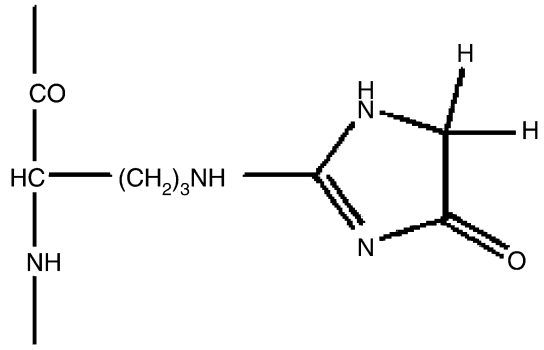
Scheme 1. Proposed mechanism of synergism between oxidative stress (i.e., MG and glyoxal) and thiamin deficiency. Thiamin deficiency causes a decrease in the activity of thiamin-dependent enzymes such as tranketolase (TK), pyruvate dehydrogenase, branched chain ketoacid dehydrogenase and α-ketoglutarate dehydrogenase (⊗). The decrease in TK activity causes a decrease in cellular NADPH levels. Under conditions of thiamin deficiency and oxidative stress the detoxification mechanisms of glyoxal and MG are decreased (×) due to lower levels of glutathione (GSH) and NADPH. The decrease in glyoxal and MG detoxification leads to an increase in their protein adducts. AKR, aldose-ketose reductase; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate and TPI, triosephosphate isomerase.

ity to oxidative stress [2]. We hypothesize that in thiamin deficiency MG and glyoxal form macromolecular adducts, and this can result in genotoxicity in colon cells. In the presence of an oxidative stress, these effects are augmented because of decreased detoxification of these aldehydes. As a first step in testing this suggestion, we examined the effect of thiamin deficiency and oxidative stress (introduced in the form of glyoxal and MG) on hydroimidazolone adduct (Scheme 1) formation in plasma proteins.

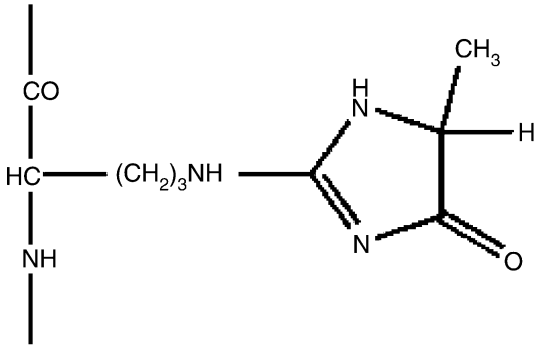
In this study, we report that reduced dietary thiamin decreased TK activity and increased α-oxoaldehyde adduct levels. The MG adduct level was not affected by oral glyoxal or MG in the animals receiving thiamin at the control levels but was markedly increased in the animals on the thiamin-reduced diet. These observations are consistent with our suggestion that the induction of ACF with marginally thiamin-deficient diets is a consequence of the formation of MG adducts and suggest that the apparent carcinogenic effect of thiamin deficiency will be increased further in animals exposed to oxidative stress.

2. Materials and methods

Glyoxal (40% w/v), MG (40% w/v), ribose-5-phosphate, 1,2-diaminobenzene, perchloric acid ($HClO_4$) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Animal diets were prepared by Research Diets Inc. (New Brunswick, NJ).



Glyoxal hydroimidazolone adduct (G-H1)



Methylglyoxal hydroimidazolone adduct (MG-H1)

Scheme 2. Predominant hydroimidazolone isomers of methylglyoxal and glyoxal.

2.1. Animals and study design

25 male Fischer 344 rats weighing 125–150 g (Harlan Sprague–Dawley) were housed in ventilated plastic cages. Care and treatment of the rats were in compliance with the guidelines of the Canadian Council on Animal Care, and the protocol was approved by the University of Toronto Animal Care Committee. The following environmental conditions applied throughout the course of the study; 12 air changes per hour, 12-h light/day cycle (lights on at 05:00 h), and an ambient temperature of 21–23 °C with a 50–60% relative humidity.

The animals were allowed to acclimatize for one week and were fed a diet of standard rat chow and water ad libitum. After one week the animals were randomized and divided into four groups: Group **A** with seven (7) animals and group **B**, **C** and **D** with six (6) animals each. The animals in group **A** were fed AIN-76 control diet containing 6 mg/kg thiamin (Research diet, New Brunswick, NJ). Group **B**, AIN-76 thiamin-deficient diet containing 1.5 mg/kg thiamin. Group **C**, AIN-76 control diet containing 6 mg/kg thiamin and 0.3% glyoxal in their drinking water and group **D**, AIN 76 thiamin-deficient diet containing 1.5 mg/kg thiamin and 0.3% glyoxal in their drinking water. A subsequent experiment was performed using 0.3% MG in the drinking water for groups **C** and **D** instead of 0.3% glyoxal.

Actual thiamin levels in the diets stored at −20 °C were measured at the end of the study by HPLC (AOAC (2000) 942.23, 986.27 Silliker Canada Co. Markham, Canada) were 5.2 and 0.9 mg/kg, respectively. Animal weight, food and water consumption was monitored weekly during the course of the experiment.

For both experiments, tail vein blood was collected at 0, 14, 28, 42 and 56 days for analysis of biochemical markers of oxidative stress (glyoxal/MG levels in the plasma) and thiamin status (TK activity in red blood cells (RBCs)). At the end of 70 days on the diets, the rats were anesthetized (CO_2 inhalation) and blood was collected by cardiac puncture for TK activity, MG and glyoxal levels and protein hydroimidazolone levels.

2.2. TK activity measurements

RBC TK activity was measured by the protocol outlined by Chamberlain et al. [19] on the blood collected via tail vein every 2 weeks and on the blood collected by cardiac puncture on day 70. Briefly, activity was measured by adding 20 μl of lysed RBCs to 200 μl reaction mixture containing 14.8 mM ribose-5-phosphate, 253 μM NADH, 185 U/ml triosephosphate isomerase and 21.5 U/ml α-GAPDH (pH 8) in Tris buffer. Fluorescence intensity was measured at $\lambda_{excitation}$ = 325 nm and $\lambda_{emission}$ = 430 nm immediately and then every 10 min for 2 h. The activity was calculated from the difference in the fluorescence intensity readings at 10 and 80 min. Percent thiamin pyrophosphate (TPP) effect (0–15% normal thiamin, 15–25% marginally deficient thiamin and >25% thiamin deficient) was calculated as outlined by Chamberlain et al. [19,20].

2.3. Glyoxal and MG levels

Glyoxal and MG levels in the plasma were measured as per the methods outlined by Okado-Matsumoto et al. [21]. A Phenomenex Luna 5μ C18 100A 250 × 4.6 mm column was used. 1,2-Diaminobenzene was used as a derivatizing agent for the analysis of glyoxal by isocratic gradient HPLC. To a 1 ml solution of plasma (1:10 dilution of plasma), 0.2 ml of 5 M $HClO_4$, 0.2 ml of 2,3-dimethylquinoxaline as an internal standard, 0.2 ml of 10 mM 1,2-diaminobenzene, and water to a 2-ml final volume. After 1 h at 25 °C, HPLC analysis was per-

Table 1
Summary of measurements for body weight at sacrifice, food and drink consumption

Group	A	B	C	D
Study 1: 0.3% glyoxal in drinking water				
Thiamin dose	6 mg/kg diet	2.5 mg/kg diet	6 mg/kg diet	2.5 mg/kg diet
Drink	Water	Water	Glyoxal	Glyoxal
Final body weight (g)	316 ± 20.81	330 ± 12.66	259 ± 18.14[a]	262 ± 20.62[a]
Food consumption (g/rat/week)	102.6 ± 16.05	108.0 ± 16.27	83.2 ± 13.94[a]	83.6 ± 12.70[a]
Water consumption (ml/rat)	n/m	n/m	135 ± 18.77	131 ± 16.31
Estimated dose (mg/kg b.w./day)	n/a	n/a	223.36	214.27
Study 2: 0.3% MG in the drinking water				
Thiamin dose	6 mg/kg diet	2.5 mg/kg diet	6 mg/kg diet	2.5 mg/kg diet
Drink	Water	Water	MG	MG
Final body weight (g)	320 ± 15.72	313 ± 6.37	320 ± 10.55	301 ± 13.93[b]
Food consumption (g/rat/week)	97.9 ± 6.71	95.6 ± 7.94	96.5 ± 5.97	92.3 ± 4.37
Water consumption (ml/rat)	119 ± 13.40	125 ± 13.19	107 ± 6.86[b]	104 ± 9.05[b]
Estimated dose (mg/kg b.w./day)	n/a	n/a	143.31	148.07

Note: n/m = not measured; n/a = not applicable.
[a] $P < 0.001$ as compared to groups A and B.
[b] $P < 0.05$ as compared to group A.

formed. The mobile phase was 50% (v/v) 25 mM ammonium formate buffer, pH 3.4, and 50% (v/v) methanol. A volume of 100 μl was injected. The flow rate was 1.0 ml/min and quinoxalines were detected at 315 nm.

2.4. Hydroimidazolone adducts measurements

Plasma levels of hydroimidazolone adducts of MG and glyoxal were measured at day 70 by quantitative immunoblotting. Dot blots were performed as per the methods outlined by Shinohara et al. [20,22]. Equal amounts of plasma protein were used for quantitative immunoblotting. Methylglyoxal-derived imidazole AGE was detected using a 1:10 000 dilution of monoclonal antibody 1H7G5 (generously donated by Dr. Michael Brownlee, Albert Einstein University, NY). Immunocomplexes were visualized using an enzyme-catalyzed fluorescence kit according to the manufacturer's instructions (Amersham, Piscataway, New Jersey) and were quantitated on a FluorChem™ 8800 (Perkin–Elmer, Ontario, Canada). A standard of bovine serum albumin (BSA) modified with MG and glyoxal was used to quantitate the dot blots as MG/glyoxal BSA equivalents.

2.5. Statistical analysis

Statistical analysis was preformed by one-way ANOVA.

3. Results and discussion

We designed this study to investigate the interactions between marginal thiamin deficiency and oxidative stress using glyoxal and MG as an exogenous model of dietary stress. It was found that reduced dietary thiamin caused a decrease in RBC TK activity and an increase in the plasma level of glyoxal and MG adducts. We also found that oxidative stress augmented the response to thiamin deficiency and caused a significant decrease in TK activity within 2 weeks. Furthermore, it caused an increase in plasma levels of MG and glyoxal at 10 weeks and increased MG and glyoxal plasma protein adducts.

Table 1 summarizes final weight as well as food and water consumption. Rats given glyoxal in the drinking water had a significant weight reduction as early as the first week under test diet (Fig. 1A) as compared to the control thiamin (5.2 mg/kg) and thiamin deficient (0.9 mg/kg) groups. This was associated with the decreased food consumption at the same time (Table 1). Rats given MG in the drinking water did not show any significant decrease in weight gain as compared to the control groups (Fig. 1B). The average dose of glyoxal and MG ingested by the animals were 57 and 45 mg/day, respectively, as assessed by the amount of drinking water consumed, and were consistent over the duration of the study. When related to body weight (b.w.) at time of sacrifice the above values correspond to an effective dose of 220 and 145 mg/kg b.w./day for glyoxal and MG, respectively.

RBC TK activity was assessed as a measure of the animal's thiamin status. To assure that the decrease observed was due to thiamin levels and not modification of the TK enzyme, the percent TPP effect (0–15% normal thiamin, 15–25% marginally deficient, >25% deficient) was calculated. We found that reduced dietary thiamin caused a decrease in RBC TK activity (~25%) within two weeks (Fig. 2A and B), which was further increased to ~35% after 4 weeks. However, the effect on the TK activity was more pronounced with the glyoxal and MG administered groups. MG and glyoxal both caused a further decrease in TK activity in the control (MG group 54% and glyoxal group 45%) and decreased-thiamin groups (MG group 69% and glyoxal group 70%). The further decrease in TK activity of the groups with MG and glyoxal could be due to the induction of oxidative stress. The TK activities of control groups in these two studies are consistent with those observed in a previous report [18]. We suggested then that thiamin deficiency (as measured by reduced TK activity) was a factor in inducing spontaneous formation of ACF in the rat. We can therefore hypothesize that further inhibition, as observed here in relation to glyoxal or MG in the diet, will enhance the carcinogenic effect. Studies are currently underway to test this interaction and the significance of the various biomarkers reported here.

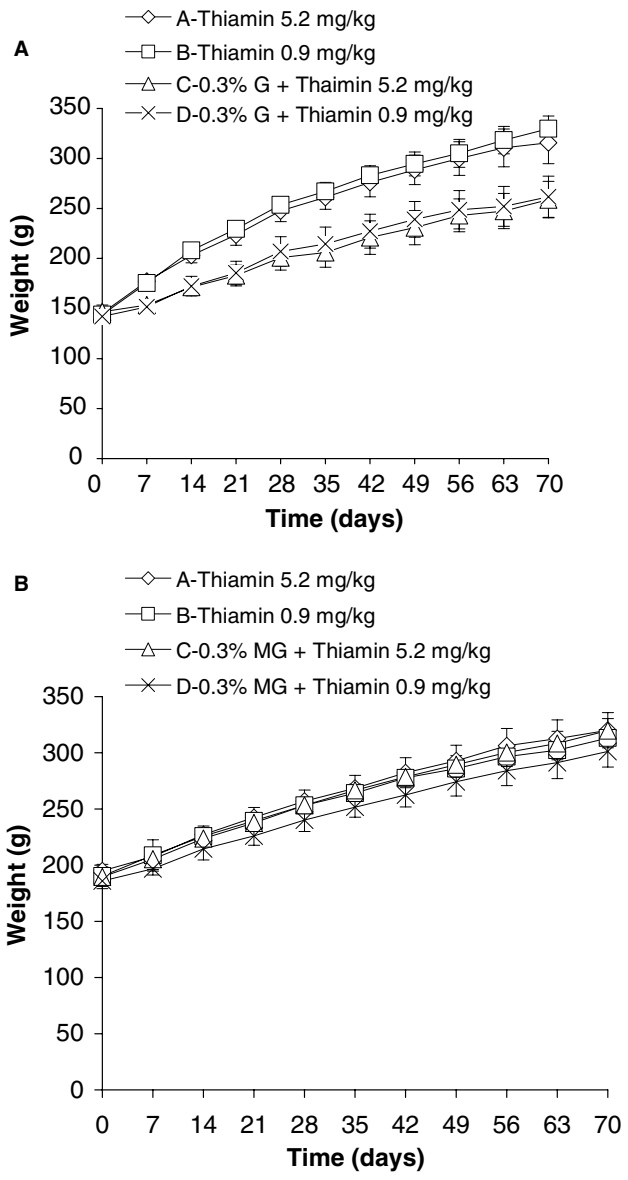


Fig. 1. Effect of glyoxal (A) or methylglyoxal (MG) (B) administration (p.o.) on rat growth rate. Here animals receiving oral glyoxal but not MG show significant reduction in weight gain after 7 days.

In our previous in vitro experiments with isolated rat hepatocyte, we found that glyoxal toxicity could be attributed to oxidative stress. As before glyoxal-induced cytotoxicity ensued there was an increase in reactive oxygen species formation, lipid peroxidation and a decrease in mitochondrial potential [8]. Thiamin has been shown to inhibit lipid peroxidation in rat

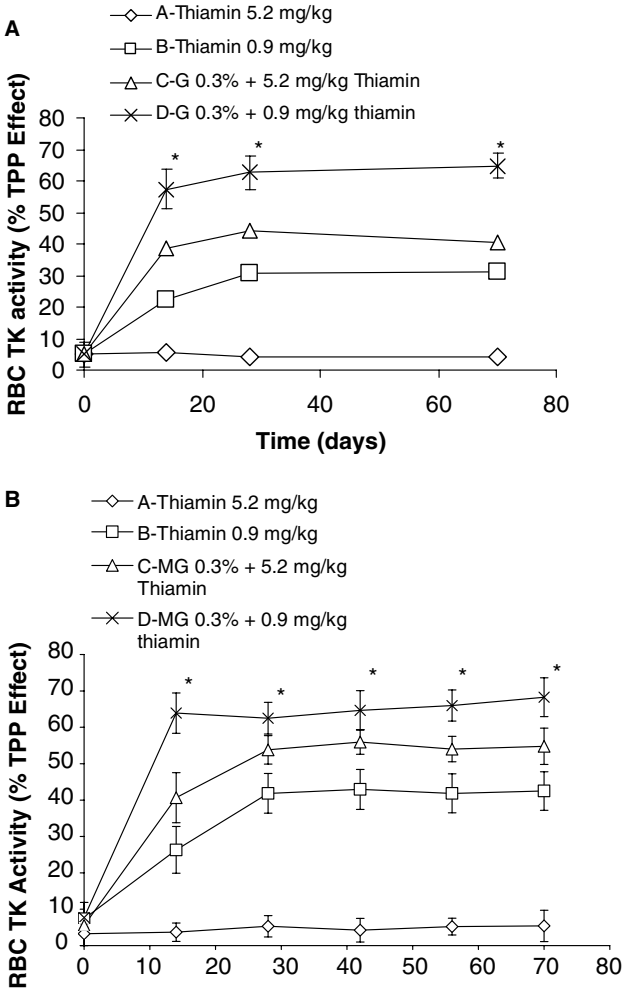


Fig. 2. Time course of RBC TK activity (A) glyoxal (G) and (B) methylglyoxal (MG) administered rats. A significant reduction in TK activity was observed at 2 weeks. $^{*}n = 6$, significant as compared to control thiamin (6 mg/kg) ($P < 0.01$). Absolute TK activity values at 70 days were 8.37, 6.40, 4.75 and 3.07 μg/mol/ml for groups A, B, C, and D, respectively, in study (A). They were 8.70, 5.29, 4.16, and 2.92 μg/mol/ml for groups A, B, C, and D, respectively, in study (B).

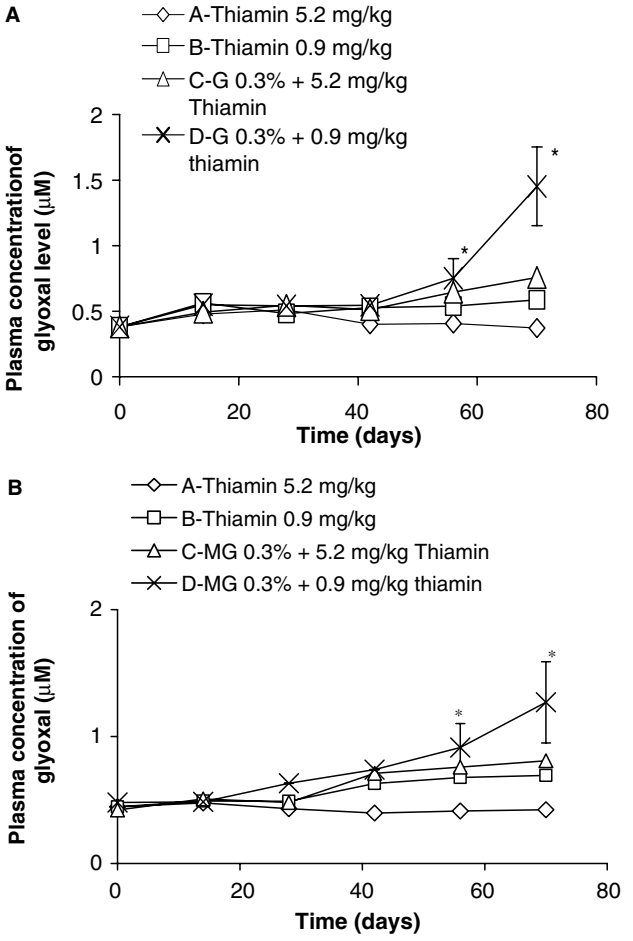


Fig. 3. Time course of plasma glyoxal (G) levels found in (A) glyoxal and (B) methylglyoxal (MG) administered rats. A significant increase was observed at 70 days. $^{*}n = 6$, significant as compared to control thiamin (6 mg/kg) ($P < 0.03$).

liver microsomes and free radical oxidation of oleic acid in vitro. Thiamin also interacts with free radicals and hydroperoxides and is oxidized to thiochrome and thiamin disulfide [23]. Thiochrome and thiamin disulfide cannot be converted to TDP and be used as a co-factor for carbohydrate metabolizing enzymes. Thiamin can act as an antioxidant in the cell by successively transferring hydrogens from the amino group of the pyrimidine ring and from the thiazole ring after ring opening [23]. Therefore, the oxidative stress caused by these reactive aldehydes could cause a decrease in thiamin which resulted in a decrease in TK activity.

As shown in Fig. 3A, there was a significant increase in plasma glyoxal levels in the group of rats that were given glyoxal in the drinking water and had decreased dietary thiamin (plasma glyoxal levels at 70 days = 1.45 μM). The same increase was observed in the group of rats given MG in the drinking water and a decreased dietary thiamin diet (plasma glyoxal levels at 70 days = 1.27 μM) (Fig. 3B). Plasma MG levels (Fig. 4A and B) for both these groups of animals were also significantly higher as compared to the control groups (MG in drinking water and decreased dietary thiamin 2.1 μM and glyoxal in drinking water and decreased dietary thiamin diet 1.64 μM) at 70 days. However, the levels of MG and glyoxal did not increase in control diet or decreased dietary thiamin diet or control diet with MG or glyoxal in the drinking water (Figs. 3 and 4). Therefore, the increase in plasma levels of MG and glyoxal observed in rats given MG or glyoxal in drinking water and decreased thiamin in the diet indicates that the metabolism of these reactive dialdehydes may be decreased. In our previous study, we found that under conditions of oxidative stress, glyoxal metabolism in hepatocyte was inhibited [8]. As mentioned earlier, thiamin deficiency and α-oxoaldehyde metabolism/oxidative stress have overlapping requirements for GSH and NADPH. GSH not only forms the primary defense mechanism of the cell against oxidative stress, but is essential for glyoxal and MG metabolism by the glyoxalase system [15]. However, when the concentration of GSH decreased below 3 mM in the liver, (normally 15 mM) the NAD[P]H dependent oxidoreductase system became the primary detoxification mechanism for these α-oxoaldehydes [24]. Thiamin deficiency by inhibiting TK affects the pentose phosphate pathway a major supplier of NADPH in the cell, which would compromise the MG and glyoxal detoxifying reductases (Scheme 1) [1].

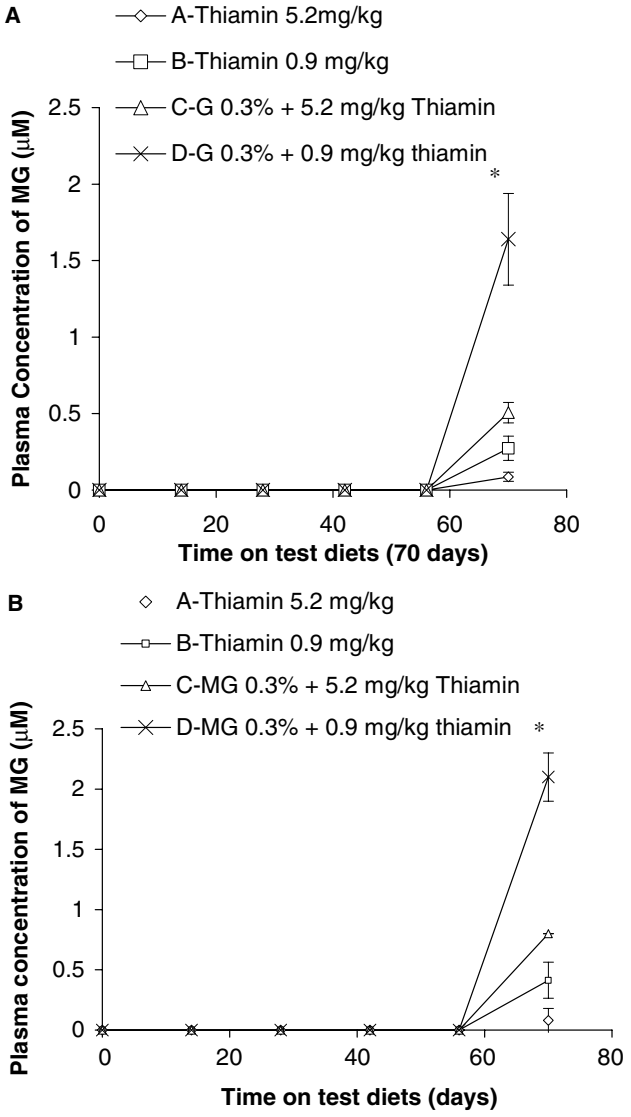


Fig. 4. Time course of plasma methylglyoxal (MG) levels found in (A) glyoxal and (B) MG groups. Significant increase was observed at 70 days. $^{*}n = 6$, significant as compared to control thiamin (6 mg/kg) ($P < 0.02$).

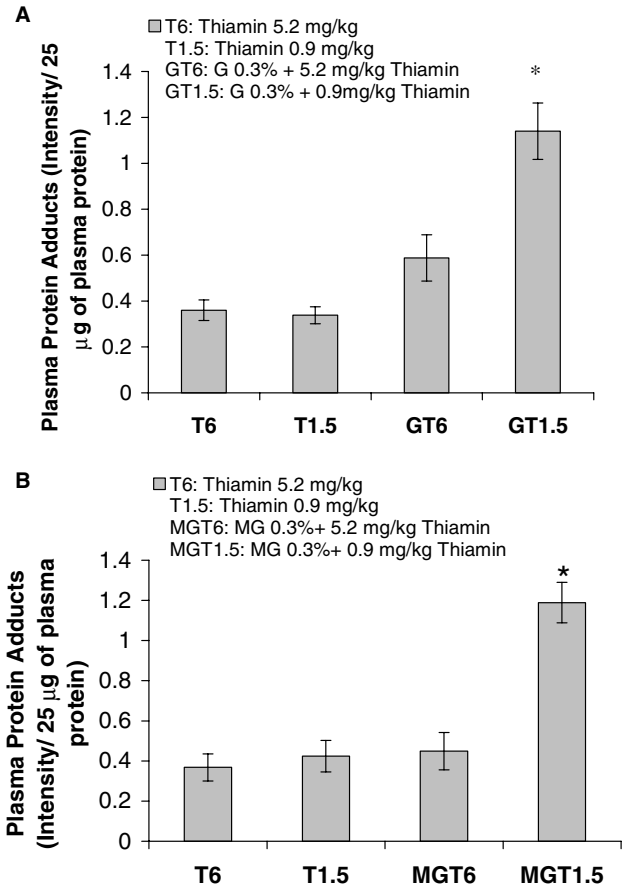


Fig. 5. Assessing plasma protein adducts of methylglyoxal (MG) and glyoxal (G) in (A) glyoxal and (B) MG administered rats at 70 days on test diets. Significant increase in adducts was observed at 70 days. Values are calculated based on BSA modified with MG. $^{*}n = 6$, significant as compared to control thiamin (6 mg/kg) ($P < 0.05$).

The increase in MG and glyoxal plasma levels was associated with an increase in the intracellular protein adducts (Fig. 5) as measured by the monoclonal antibody against MG/glyoxal hydroimidazolone adduct. A significant increase in adducts was observed at 70 days in the rats with thiamin deficiency and oxidative stress (MG group = 1.19 and glyoxal group = 1.14 BSA equivalent). In a similar study, we looked at the effects of *N*-acetylcysteine supplementation (precursor for GSH formation and antioxidant) on the level of these protein adducts under conditions of thiamin deficiency and oxidative stress. We found that the level of adducts in the groups given 0.3% *N*-acetylcysteine (in the drinking water) decreased approximately 3-fold under conditions of thiamin deficiency alone or glyoxal/MG-induced oxidative stress (paper in preparation). We also currently have studies underway with *N*-acetylcysteine plus thiamin deficiency and MG/glyoxal as exogenous sources of oxidative stress to further establish this relationship between the increase in plasma hydroimidazolone adducts and increased ACF formation.

The effect of these hydroimidazolone adducts in vivo is unknown [10]. There is in vitro evidence that hydroimidazolone formed from MG is a recognition factor for the binding of human serum albumin minimally modified with MG (MG_{min}-HSA) to monocytic THP-1 cells [25]. MG_{min}-HSA induces synthesis and secretion of interleukin-1β and macrophage colony-stimulating factor from peripheral human monocytes in vitro, as well as inducing tumor necrosis factor-α synthesis and secretion from THP-1 cells [26,10]. These protein adducts may be used as a biomarker of oxidative stress and colon carcinogenesis.

Scheme 1 shows a mechanism proposed for the relationship between thiamin deficiency, oxidative stress (i.e., MG and glyoxal) and the formation of the adducts. The mechanism explains the results of our previous in vitro study demonstrating that glyoxal increases cytotoxicity of isolated rat hepatocytes under thiamin deficient conditions, assuming that the glyoxal adducts are cytotoxic [2]. It also explains our preliminary results indicating that marginally thiamin deficient diets induce the formation of ACF, suggesting that the glyoxal adducts can be genotoxic and carcinogenic [18]. That is, suggesting oxidative stress can reduce the concentration of thiamin and that oxidative stress with thiamin deficiency increases intracellular glyoxals, cytotoxicity,

genotoxicity and carcinogenicity. This could explain the association of oxidative stress and colon carcinogenesis in colonic inflammation as in ulcerative colitis, with the consumption of foods containing iron and heme such as meats and, perhaps, in the carcinogenesis process itself as in ACF [27].

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